AD-A158 414 RADIOMETRIC METHODS FOR RAPID DIAGNOSIS OF VIRAL INFECTION(U) JOHNS HOPKINS UNIV BALTIMORE MD H N WAGNER ET AL. NOV 75 DAMD17-75-C-5041 1/1

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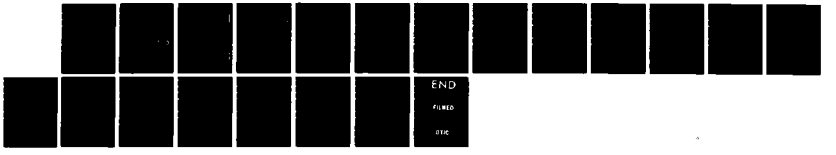

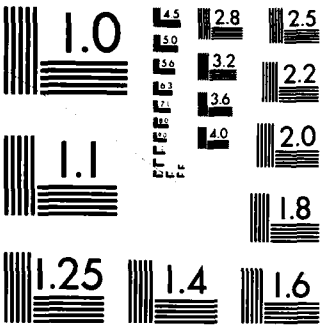

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RADIOMETRIC METHODS FOR RAPID DIAGNOSIS OF VIRAL INFECTION

Annual Report

Henry N. Wagner, Jr., M.D.

November 1975

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-75-C-5041

The Johns Hopkins University
Baltimore, Maryland 21218

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER

2. GOVT ACCESSION NO. AD-A158 414

3. RECIPIENT'S CATALOG NUMBER

4. TITLE (and Subtitle)

RADIOMETRIC METHODS FOR RAPID DIAGNOSIS OF VIRAL INFECTION.

5. TYPE OF REPORT & PERIOD COVERED
Annual Report
2/1/75--1/31/76

6. PERFORMING ORG. REPORT NUMBER

7. AUTHOR(s)

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Patricia Charache, M.D.

8. CONTRACT OR GRANT NUMBER(s)

DAMD-17-75-C-5041

9. PERFORMING ORGANIZATION NAME AND ADDRESS

The Johns Hopkins University
34th and Charles Streets
Baltimore, Maryland 21218

10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS

61102B.3M161102BS03.00.043

11. CONTROLLING OFFICE NAME AND ADDRESS

Command
U.S. Army Medical Research and Development/
Fort Detrick, Frederick, MD 21701-5012

12. REPORT DATE
November 1975

13. NUMBER OF PAGES
19

14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)

15. SECURITY CLASS. (of this report)
Unclassified

15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release;
distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Radiometric methods
Virus

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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20. respectively. Infected cells showed a 23 to 26% reduction in glucose-1-^{14}C oxidation and 355 to 498% increase in DNA synthesis four to six hours postinfection as compared to uninfected control cells. These changes in cellular metabolism were observed 14 hours before visible signs of cytopathic effects. The increase in DNA synthesis was completely inhibited by viral neutralization with herpes simplex antiserum. Increased DNA synthesis was observed five hours postinfection with 10^4 to $10^{6.5}$ $TCID_{50}$ units of virus. The radiometric methods for the detection of viral effect on cellular metabolism are simple, fast, and objective.

TABLE OF CONTENTS

ABSTRACT

Two radiometric techniques were developed for detecting the presence of herpes simplex virus type 1 in stationary monolayers of the diploid cell line WI-38. The time of detection was compared to that obtained from visual examination for cytopathic effects in the same cell line. Glucose-1-^{14}C oxidation and DNA synthesis of infected and uninfected cells were determined by $^{14}CO_2$ production measured by an ionization chamber, and ^{3}H-thymidine incorporation measured by scintillation counting, respectively. Infected cells showed a 23 to 26% reduction in glucose-1-^{14}C oxidation and 355 to 498% increase in DNA synthesis four to six hours postinfection as compared to uninfected control cells. These changes in cellular metabolism were observed 14 hours before visible signs of cytopathic effects. The increase in DNA synthesis was completely inhibited by viral neutralization with herpes simplex antiserum. Increased DNA synthesis was observed five hours postinfection with 10^{4} to $10^{6.5}$ $TCID_{50}$ units of virus. The radiometric methods for the detection of viral effect on cellular metabolism are simple, fast, and objective.

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INTRODUCTION

While detection of bacteria is commonplace in clinical medicine, detection of viruses remains difficult and has not achieved the widespread use that is warranted by the frequent occurrence of viral infections. There is a need for rapid and simplified techniques for detecting virus in clinical materials to facilitate the management of patients. Currently used methods such as immunofluorescent (1, 2) and immunoenzymatic (3, 4) techniques are rapid, but require carefully controlled conditions and experienced personnel. The development of a simple, rapid, and objective method would have an obvious advantage in diagnostic virology.

It is well documented that numerous biochemical changes and functional disturbances precede visual histological changes and cell death in cell cultures infected with a cytocidal virus such as herpes simplex type 1 (HSV-1) (5). Microscopic evidence of cellular infection (cytopathic effect) is the diagnostic criterion most commonly employed in the clinical laboratory for the detection of viral presence (6, 7). It is our hypothesis that early biochemical effects of viral growth in cell culture can be used as a means for detecting viral presence, the same basic principle having been successfully employed in the detection of bacterial growth (8, 9). Infection of human embryonic lung fibroblasts, WI-38 cells, by HSV-1 was used as the test model. In this study we report the radiometric measurement of the effects of HSV-1 on glucose oxidation and nucleic acid synthesis by WI-38 cells during early hours of infection before any visible signs of abnormal cell function are apparent. The results are promising for further development of radiometric methods for the detection of viruses.

MATERIALS AND METHODS

Cells. WI-38 cells in the 20th to 24th passage (HEM Research, Inc., Rockville, Md.) were planted at a concentration of 1×10^5 cells/ml in a total volume of 2 ml and grown as stationary monolayers (two to four days) in basal medium Eagle, Earle's base plus 10% fetal calf serum, 25mM herpes buffer, 100 U of patassium penicillin G per ml, 100 ug of streptomycin per ml, and 100 ug of kanamycin per ml. The glucose oxidation method (GO) utilized sterile 10 ml serum vials plus rubber liners (Johnston Laboratories, Inc., Cockeysville, Md.), and aluminum airtight seals (Wheaton Scientific, Millville, N. J.)

for cultivation, the vials being incubated in a horizontal position at 37° C. The nucleic acid synthesis method (NAS) utilized autoclaved 20 ml glass liquid scintillation vials plus non-toxic screw caps (Wheaton Scientific) incubated in a vertical position. Both cell systems yielded 3-4 x 10^5 cells/monolayer routinely, as determined by direct cell counting as follows: the monolayer was trypsinized with 1.0 ml of 0.25% trypsin/ 0.02% EDTA (Gibco, Grand Island, New York), neutralized with 0.9 ml of medium, stained with 0.1 ml of a 0.4% trypan blue aqueous solution, and counted via a counting chamber.

Virus stock. A patient isolate of HSV-1 was obtained from The Johns Hopkins Hospital Virology Laboratory, Baltimore, Md. Stock virus was prepared in WI-38 monolayers maintained on Eagle's minimal essential medium, Earle's base plus 3% fetal calf serum with buffer and antibiotics as listed for growth medium. Virus-cell material was frozen and thawed twice and then stored at -70° C. The stock was sterility tested and assayed for glucose concentration. The titer was determined by tube titration in WI-38 cells and the 50% endpoint calculated by the Reed and Muench method (10), yielding 7 x $10^{6.5}$ or 3 x 10^6 $TCID_{50}$/0.1 ml. Tube titration was confirmed by plaque assay (11) which yielded 7 x 10^6 plaque forming units/0.1 ml.

Glucose assay. Virus stock was mixed 1:1 with 10% trichloroacetic acid and centrifuged at 200g for 20 minutes. The supernatant was used to measure the glucose concentration by hexokinase-glucose-6-phosphate dehydrogenase method utilizing an automatic clinical analyzer (Dupont Instruments, Newton, Conn.). The glucose concentration of the virus stock was found to be 36 $\pm$ 1 mg%.

Glucose oxidation system. Serum vials containing preconfluent monolayers with overlay medium removed were infected with 0.1 ml of virus stock, at a virus-to-cell ration of approximately 10:1. Uninfected cells received 0.1 ml of an equalized glucose solution prepared in Earle's basal salts. Virus was exposed to the cells for 60 minutes at 37° C with mold agitation at 15 minute intervals. Following the adsorption period, 1.9 ml of low glucose maintenance medium (maintenance medium without the usual 0.1% glucose) plus 4 uCi of D-glucose-1-^{14}C (Amersham-Searle Corp., Arlington Heights, Ill., 60 mCi/mM) was added per vial. All vials were prepared in quintuplet. Background controls consisted of an equal volume of ^{14}C-labeled medium without cells or virus.

Measurement of glucose oxidation. The $^{14}CO_2$ produced by cellular metabolism was measured with the Bactec R-301 (Johnston Laboratories, Inc.). The $^{14}CO_2$ produced from the ^{14}C-substrate in the culture vial was flushed into the ionization chamber through two needles inserted in the septum and measured as ionic current. This measured radioactivity is expressed as "index units" (I. U.) where 100 I. U. $\simeq$ 0.025 uCi. The atmosphere in the vial was replaced with 10% CO_2 culture gas. The $^{14}CO_2$ production from infected and uninfected cells was monitored at 2, 4, 6, 24, 48, and 72 hours postinfection, infection time beginning when the ^{14}C-labeled medium was added.

Nucleic acid synthesis system. Stationary monolayers were infected as in GO system. 0.1 ml phosphate buffered saline (PBS) was added to uninfected control cells. Following the adsorption period, 1.9 ml of maintenance medium plus 1 uCi of methyl-^3H thymidine (52 Ci/mM) or 5, 6-^3H uridine (40 Ci/mM, Amersham-Searle Corp.) was added to each infected and control vial. Background controls consisted of equal amounts of ^3H-labeled medium without cells or virus. All samples were prepared in duplicate and taken for measurement at 1, 2, 4, and 6 hours postinfection.

Viral neutralization tests were performed as follows: herpes simplex human immune serum (Flow Laboratories, Rockville, Md.) was heat inactivated at 56° C for 30 minutes, mixed 1:1 with the virus stock, incubated at 37° C for 60 minutes, and 0.2 ml of the mixture inoculated. Samples were assayed at 1, 2, 4, and 6 hours postinfection.

Virus stock was also diluted in maintenace medium to contain $\log_{10}$ quantities for dose response determination. 0.1 ml of the appropriate dilutions was added per vial. Uninfected control cells received 0.1 ml maintenance medium. In this group of experiments, the cells were cultured in one dram vials (Wheaton Scientific) which yielded 4×10^4 cells per vial as compared to $3\text{-}4 \times 10^5$ cells per vial in previous experiments. Samples were prepared in triplicate and assayed 5 hours postinfection.

Measurement of nucleic acid synthesis. The amount of ^3H-thymidine or ^3H-uridine incorporation by WI-38 cells was measured by liquid scintillation counting. At the designated time intervals, the ^3H-labeled medium was aspirated, the remaining cell monolayer or empty vial in case of background controls without cells was washed twice with 10 ml of cold PBS. Preliminary experiments indicated that this washing procedure did not detach cells as determined by direct cell counting and removed all of the extracellular radioactivity as measured with ^{14}C-Inulin. Samples were prepared for liquid scintillation

counting by the addition of 0.4 ml Protosol (0.5 M, New England Nuclear, Boston, Mass.) for ten minutes at 37° C, followed by 15 ml of Bray's solution. Samples were counted with a Packard Tri-Cab scintillation spectrometer model 3003 (Packard Instrument Co., Downers Grove, Ill.).

Examination for cytopathic effects. Radiometric viral detection in GO and NAS systems was compared with visual detection by typical cytopathic effects (CPE) in WI-38 test tube monolayers maintained in triplicate. After removal of the overlay medium, virus was inoculated and tubes placed on a roller for 60 minutes at 37° C. 1.0 ml of maintenance medium was then added, and microscopic examination for CPE was performed at comparable time intervals, continuing until positive CPE was identified.

Sterility testing. Sterility checks of random samples from each experiment were performed on chocolate agar, in Schaedler's broth, and radiometrically with glucose-U-^{14}C aerobic and anaerobic culture vials (Johnston Laboratories, Inc.).

Statistics. The statistical significance was calculated based on pair differences (12).

RESULTS

Effect of HSV-1 on glucose oxidation by WI-38 cells. The effect of HSV-1 on glucose-1-^{14}C oxidation by WI-38 cells is shown in Table 1. As early as 2 hours after infection there was a significant depression of glucose-1-^{14}C oxidation by HSV-1 infected cells. This effect was observed 16 hours before visual signs of CPE. The degree of inhibition of glucose-1-^{14}C oxidation by HSV-1 infected cells continued to increase up to 72 hours (17% at 2 hours vs. 43% at 72 hours).

Effect of HSV-1 on nucleic acid synthesis by WI-38 cells. Table 2 shows the effect of HSV-1 on DNA synthesis by WI-38 cells. In virus infected cells, there was a marked stimulation of DNA synthesis. At 4 hours postinfection, there was a 4-fold stimulation. This was at least 14 hours before any signs of CPE were visible. In contrast, HSV-1 had no effect on RNA synthesis by WI-38 clels (Table 3). Thus, the tremendous stimulation of DNA synthesis by this DNA virus was not accompanied by a comparable stimulation in RNA synthesis by WI-38 cells.

Addition of 1×10^4 bacteria per sample of three different bacterial species (Staphylococcus epidermidis, Pseudomonas aeruginosa, and Acinetobacter caloaceticus var. anitratus) had no effect on the DNA synthesis of HSV-1 infected or control cells.

Effect of viral neutralization with specific antiserum on DNA synthesis by WI-38 cells. Neutralization of HSV-1 with specific antiserum resulted in complete elimination of the stimulation of DNA synthesis observed when HSV-1 alone is present (Fig. 1). Antiserum alone had no effect on DNA synthesis by WI-38 cells. Neutralization of virus with antiserum was confirmed by the absence of CPE over a three day period.

Effect of various numbers of HSV-1 on DNA synthesis by WI-38 cells. So far, 3×10^6 $TCID_{50}$ units of HSV-1 and $3\text{-}4 \times 10^5$ WI-38 cells per sample were used in each experiment. In order to determine the relative sensitivity of the NAS system, various numbers of virions ranging from 1×10^1 to 3×10^6 $TCID_{50}$ units were tested. Preliminary experiments indicate that an increase in the multiplicity of infection by a decrease in the number of WI-38 cells increases the degree of stimulation of DNA synthesis. Therefore, monolayers composed of 4×10^4 WI-38 cells were used for this part of the study. As shown in Table 4, a significant stimulation of DNA synthesis by WI-38 cells could be detected with 1×10^4 virions at 5 hr after infection.

DISCUSSION

The results of this study indicate that radiometric measurements of the effect of HSV-1 on the metabolism of tissue culture cells can be used as means of viral detection, and neutralization of virus with specific antiserum can be used for the purpose of speciation. This effect on cellular metabolism could be detected as early as 5 hr postinfection of 4×10^4 WI-38 cells with 1×10^4 $TCID_{50}$ units of virus. This is at least 13 hr before any evidence of visible cytopathic effects.

The sequence of replication and biosynthesis of HSV and its effect on host macromolecular synthesis have been well documented (13,14). There is an early and complete shutdown of host DNA synthesis within 3 to 5 hr after infection, indicating that the large increase of DNA synthesis observed by HSV-1 infected cells is due to the replication of the viral genome. Only about 20% of the viral DNA is found in infectious particles (13). Thus, the measurement of nucleic acid synthesis would seem more sensitive than methods that depend on the presence of the infectious virus.

The early inhibition of glucose-1-C oxidation by HSV-1 in WI-38 cells has not been previously demonstrated. Graves (15), utilizing a poliovirus-Hela cell system, was unable to demonstrate any effect on glucose oxidation. Our method of measurement of glucose oxidation by monitoring $^{14}CO_2$ release with an ionization chamber has the advantage of

being non-destructive and allows repeated sampling from the same vial over extended periods. However, the presence of a relatively high concentration of glucose in biological fluid such as blood, poses a practical problem since this non-labeled glucose would compete with ^{14}C-glucose for oxidation.

In the nucleic acid synthesis system, the cells were grown in liquid scintillatation ccunting vials. This allowed us to perform the entire procedure, from infection of virus to the final quantification of DNA synthesis, in the same vial. These radiometric techniques for the early detection of HSV-1 are simple and objective. Further study is necessary to assess application of these techniques to the actual detection and identification of the virus in clinical specimens.

TABLE 1. EFFECT OF HSV-1 ON GLUCOSE-1-^{14}C OXIDATION BY WI-38 CELLS*

	Time (hours after infection)					
	2	4	6	24	48	72
Control	58 ± 8	226 ± 36	438 ± 74	1222 ± 203	1874 ± 253	2310 ± 253
HSV-1	44 ± 8	173 ± 33	326 ± 33	720 ± 125	1078 ± 168	1322 ± 187
% Control	83.4	76.6	74.4	59.0	57.5	57.2
P value	<0.01	<0.01	<0.005	<0.01	<0.005	<0.005

* The results are expressed as mean ± standard error of the mean (index units) of the cumulative $^{14}CO_2$ production. The number of WI-38 cells used was 3-4 x 10^5 and the quantity of HSV-1 was 3 x 10^6 $TCID_{50}$ units. Each experiment was done in quintuplet and the results were averaged. Number of experiments: 4.

TABLE 2. EFFECT OF HSV-1 ON DNA SYNTHESIS BY WI-38 CELLS*

	Time (hours after infection)			
	1	2	4	6
Control	2958 ± 678 (4)+	3700 ± 447 (4)	5818 ± 685 (5)	7245 ± 720 (3)
HSV-1	2789 ± 479 (4)	8341 ± 1930 (4)	26,482 ± 3392 (5)	43,298 ± 7882 (3)
% Control	94.3	225.4	455.2	597.6
P value	>0.5	<0.1	<0.005	<0.05

* The results are expressed as mean ± standard error of the mean (cpm). The number of WI-38 cells used was 3-4 x 10^5 and the quantitiy of HSV-1 was 3 x 10^6 $TCID_{50}$ units. Each experiment was done in duplicate and the results averaged.

+ Number in parenthesis indicates number of experiments.

TABLE 3. EFFECT OF HSV-1 ON RNA SYNTHESIS BY WI-38 CELLS*

	Time (hours after infection)			
	1	2	4	6
Control	5242 ± 2475	10,086 ± 4324	17,007 ± 8498	28,328 ± 11,607
HSV-1	5305 ± 2503	10,158 ± 4610	20,274 ± 9392	30,351 ± 13,603
% Control	101.2	100.7	119.2	107.1
P value	> 0.5	> 0.5	> 0.1	> 0,4

* The results are expressed on the same basis as Table 2. Number of experiments: 3.

TABLE 1. EFFECT OF VARIOUS NUMBERS OF HSV-1 ON DNA SYNTHESIS BY WI-38 CELLS 5 HOURS POSTINFECTION*

	Infectivity ($TCID_{50}$/Sample)						
	$10^{6.5}$	10^{6}	10^{5}	10^{4}	10^{3}	10^{2}	10^{1}
% Control	844 ± 79 (3)+	741 ± 12 (3)	437 ± 65 (4)	198 ± 14 (4)	117 ± 17 (4)	126 ± 7 (3)	125 ± 17 (4)
P value	<0.025	<0.001	<0.025	<0.01	>0.2	>0.2	>0.2

* The results are expressed as mean ± standard error of the mean of percent control (cpm). Each experiment was done in triplicate and the results were averaged. The number of WI-38 cells used as 4×10^4.

+ Number in parenthesis indicates number of experiments.

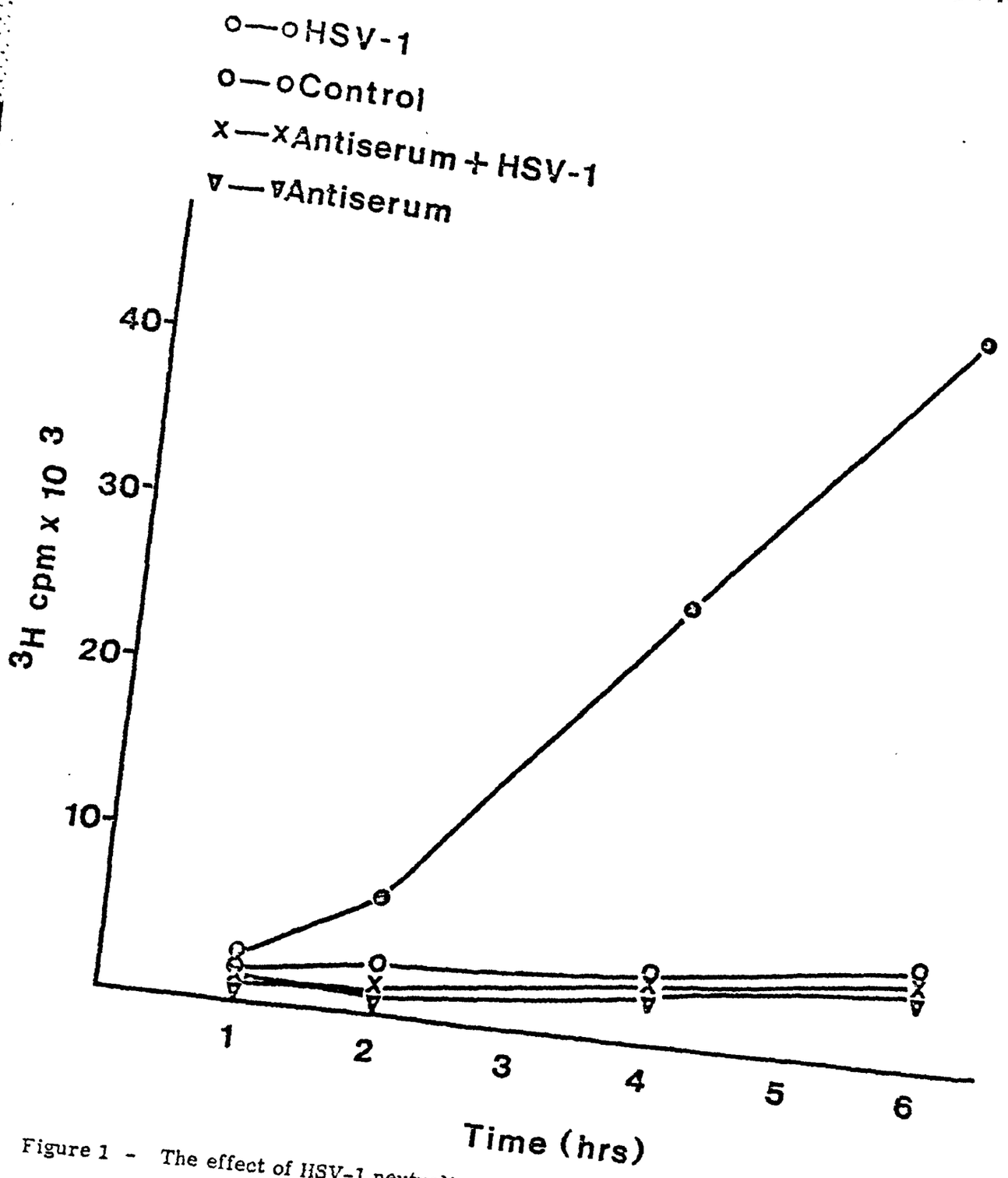

Figure 1 - The effect of HSV-1 neutralization by specific human immune serum on DNA synthesis by WI-38 cells. Each point represents the mean of duplicate samples from a typical experiment.

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Figure 1 - The effect of HSV-1 neutralization by specific human immune serum on DNA synthesis by WI-38 cells. Each point represents the mean of duplicate samples from a typical experiment.

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